

ORIGINAL ARTICLE

Ambrosin sesquiterpene lactone exerts selective and potent anticancer effects in drug-resistant human breast cancer cells (MDA-MB-231) through mitochondrial mediated apoptosis, ROS generation and targeting Akt/ β -Catenin signaling pathway

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Summary

Purpose: Breast cancer accounts for a significant proportion of cancer burden among women world over. Concerning breast cancer treatment, there are only few chemotherapeutic agents available, which also have serious side effects. The present study was thus designed to explore in vitro the antitumor effects of ambrosin sesquiterpene lactone against human drug-resistant breast cancer cells (MDA-MB-231).

Methods: WST-1 assay was used to determine cell viability. The fact that ambrosin induced apoptosis was studied through acridine orange (AO)/ethidium bromide (EB) staining using fluorescence microscopy as well as using flow cytometry in association with annexin-v/propidium iodide (PI) staining. Furthermore, western blot assay was used to study effects of ambrosin on apoptosis-related protein expressions including Bax and Bcl-2, as well as to study the effects on numerous caspases and Akt/ β -Catenin Signaling Pathway. The effects on reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were evaluated by flow cytometry.

Results: The results showed that ambrosin with an IC₅₀ value of 25 μ M decreased the viability of the MDA-MB-231 cells. The cytotoxicity of ambrosin was also investigated on the MCF-

12A normal breast cells which showed that it exerted very low toxic effects on these cells. Ambrosin also caused remarkable changes in the morphology and suppressed the colony forming potential of MDA-MB-231 cells. The AO/EB staining assay showed that ambrosin inhibits the viability of cancer cells via induction of apoptotic cell death which was associated with increase in Bax and reduction in Bcl-2 levels. The apoptotic cells increased from 3.5% in the controls to around 56% at 50 μ M concentration in the MDA-MB-231 cells. It was also seen that ambrosin treatment to these cancer cells resulted in substantial suppression in MMP and remarkable rise in ROS in a dose-dependent manner. This molecule also significantly inhibited the Akt/ β -catenin signalling pathway by reducing the expressions of phosphorylated GSK-3 β and Akt.

Conclusions: Taken all together, the results of our study indicate that ambrosin sesquiterpene may be developed as a promising anticancer agent in human breast cancer provided further in-depth studies are performed.

Key words: ambrosin, breast cancer, apoptosis, western blot, fluorescence microscopy

Introduction

Breast carcinoma (BC) is a lethal neoplastic disorder, associated with high mortality among females worldwide [1]. BC incidence shows an

alarming increase in USA, China, Malaysia and many Asian countries from the previous three decades, with a 3.1% increase annually. Over 1.6

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Received: 16/03/2020; Accepted: 05/04/2020

million new cases with this malignancy were registered worldwide in 2010 and 2012 [2,3]. BC is both histopathologically and genetically a heterogeneous disease and the underlying mechanisms of disease progression still remain uncertain [4]. Distinctive subtypes of BC are demarcated on key expressions of hormone receptors and HER2 like triple-negative, HER2 and luminal subtypes [5]. These subtypes are due to several gene mutations in basal or luminal progenitor cells, resulting in poor diagnosis and treatment as well as showing different metastatic pattern, biology and treatment approaches [6]. BC has limited treatment options with lower effectiveness due to side effects and disease relapse including surgery, conventional chemotherapy or radiation therapy. Natural products have served as a key source of chemotherapeutic agents due to their impressive bioactivity profiles and around 70% of the currently used anticancer drugs are either natural products or compounds related to them [7-12]. Sesquiterpene lactones are a class of naturally occurring secondary metabolites, frequently found in plants and bear a α -methylene- γ -lactone ring [13]. Presence of α -methylene- γ -lactone ring enhances their alkylating property, thus are thought to alkylate nucleophiles within the cell, like proteins with sulfhydryl groups. One of the specified targets of sesquiterpene lactones is p65 - a member of heterodimeric transcription factor NF- κ B - which controls an array of bio-activities like cell survival, development, proliferation, immune responses, angiogenesis, metastasis and invasion [14,15]. Ambrosin is a pseudoguaienolide sesquiterpene lactone found in large numbers of ragweed species around the world [16,17]. Ambrosin is a nonpolar compound and is devoid of any phenolic moiety. It has been reported with various pharmacological and biological activities including anticancer and potent NF- κ B inhibition [18,19]. Furthermore, the molecular, behavioural and immunohistochemical experiments have revealed Ambrosin's inhibitory effects on amyloid genesis, neuro-inflammation and neuron death [20].

The current study was designed to evaluate Ambrosin for its selective and potent anticancer effects in drug-resistant MDA-MB-231 BC cells through mitochondrial mediated apoptosis, ROS generation and targeting Akt/ β -Catenin signalling pathway.

Methods

Cell viability determination

For cell viability estimation WST-1 assay was performed. MDA-MB-231 BC cells and normal MCF-12A epithelial breast cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

Briefly, 200 μ l of Dulbecco's Modified Eagle's Medium (DMEM) was placed in 96-well plates and both the cell types were cultured at a density of 3.2×10^5 cells/well. Cells were treated with different doses of Ambrosin like 0.78, 1.56, 3.12, 6.24, 12.5, 25, 50, 100 and 200 μ M, in a 5% CO₂ incubator at 37°C for 72 h. Vehicle control was regulated by DMSO. Afterwards, treated cells in each well plate were subjected to treatment with 10 μ l of WST-1 cell viability reagent [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2Htetrazolium] (Clontech Laboratories, Inc., Mountain View, CA, USA). Cells were then mixed properly with WST-1 reagent through shaking for 2-3 min followed by incubation at 37°C for a time interval of 3 h. Finally, each well was observed with a microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) and absorbance was estimated at a wavelength of 430 nm.

Cell morphology determination via acridine orange (AO)/ ethidium bromide (EB) staining

MDA-MB-231 BC cells were harvested through trypsinization on reaching 80% confluence with a density of 4.5×10^5 cells per well in a 24-well cell culture plates with cover slips. Harvested cells were subjected to Ambrosin treatment at changing concentrations (control, 12.5, 25 and 50 μ M) and incubated in 5% CO₂ incubator at 37°C for 24 h. Prior to staining with 10 μ L AO/EB staining solution, Ambrosin treated cells were fixed using formaldehyde (4%). Finally, cells were observed and studied under fluorescence microscope (Olympus Co., Tokyo, BX51TRF, Japan).

Apoptosis analysis through Annexin V-FITC/PI double staining

Apoptotic cell percentage was quantified through Annexin V-FITC/PI double staining. Briefly, MDA-MB-231 BC cells were put onto 6-well plates at a concentration of 3×10^4 cells per well. Seeded cells were then exposed to varying doses of Ambrosin (control, 12.5, 25 and 50 μ M) and harvested. Thereafter, cells were counted using TC10 Cell Counter (Bio-Rad, USA) followed by labelling with Annexin-V-FITC/PI (Beyotime, China) in accordance with the manufacturer's guidelines. Labelled/stained cells were examined through flow cytometer (BD, Bioscience FACSCalibur) at approximate fluorescence excitation maxima of 480 and 540 nm.

Phase contrast microscopy

Phase contrast microscopy was performed to observe morphological changes in MDA-MB-231 BC cells. Briefly, 4×10^4 cells were incubated with the target molecule (Ambrosin) for 48 h at varying concentrations (control, 12.5, 25 and 50 μ M) in 60 mm diameter tissue culture dishes. Total DMEM was decanted and cells were washed with PBS. Finally, using phase contrast inverted microscope (Leica DMI 3000B, Germany), morphological changes were detected at 200x magnification.

Clonogenic assay

For clonogenic assay, MDA-MB-231 BC cells were harvested at the exponential growth phase and collected

through hemocytometer. Seeding of cells was performed at a concentration of 360 cells per each well. The cells were incubated for attachment for 48 h. Afterwards, the cells in each well were subjected to Ambrosin treatment at varying concentrations (control, 12.5, 25 and 50 μM). Drug treatment was followed by incubation for 96 h. Washing was performed with phosphate-buffered saline (PBS) and fixation with methanol. Finally, fixed cells were stained with crystal violet for 30 min and examined under a light microscope.

Estimation of reactive oxygen species and mitochondrial membrane potential (ROS and MMP)

Cell culturing of MDA-MB-231 breast cancer cells was done in 6-well plates which thereafter were exposed to Ambrosin with changing concentrations (control, 12.5, 25 and 50 μM). For ROS percentage calculation, cells were collected and 10 μM of DCFH-DA (Dichlorodihydro-fluorescein diacetate) working solution with serum-free liquid was added. Afterwards, MDA-MB-231 breast cancer cells were incubated at a temperature of 37°C for 30 min. The mixture was replaced every 3-6 min so that cells and probe were in full contact. Prior to investigation through flow cytometry (BD, Bioscience FACSCalibur) cells were washed twice with PBS.

For MMP calculation, cells were cultured and treated as above and then collected in DMEM (0.5 ml) (Dulbecco's Modified Eagle's Medium). Ambrosin-treated cells were then stained with JC-1 solution and mixed well before incubating for 20 min at 37°C. Afterwards, cells were centrifuged for 10 min and the supernatant was discarded. The pellet was then washed twice with JC-1 staining buffer (1 \times) followed by re-suspension in JC-1 staining buffer (0.5 ml). Finally, cells were examined through flow cytometry (BD, Bioscience FACSCalibur).

Western blotting analysis

MDA-MB-231 BC cells were exposed to varying doses of the molecule (control, 12.5, 25 and 50 μM) followed by lysing with lysis buffer RIPA. About 40 μg of proteins from each lysate were separated and then shifted to polyvinylidene difluoride (PVDF) membranes. Blocking of the membranes was performed at 25°C for

1 h with fat-free milk. Next, cells were treated with primary antibody at 4°C overnight. Accordingly, cells were treated with secondary antibody and incubated. Finally, Odyssey infrared imaging system was used for signal detection and Actin was used for normalisation.

Statistics

All the data are shown as mean \pm SD. The significance levels for assessment of differences were obtained with a one-way ANOVA, and after that Bonferroni and Dunnett *post hoc* tests were used for multiple comparisons (Graph-Pad Software, USA). In comparison to control, $p < 0.05$ was considered statistically significant.

Results

Induction of cytotoxicity by Ambrosin in drug-resistant MDA-MB-231 BC cells and normal MCF-12A epithelial breast cells

The effect on cell viability of drug-resistant MDA-MB-231 BC cells and normal MCF-12A epithelial breast cells by Ambrosin were evaluated by WST-1 cell viability assay which showed that the viability of cancer breast cells was declined remarkably on increasing the dose concentration (0.78, 1.56, 3.12, 6.24, 12.5, 25, 50, 100 and 200 μM). The cell viability was observed to decline from 100% to about 5% after treatment (Figure 1A). The cell viability of normal MCF-12A epithelial breast cells was much less affected showing lesser cytotoxicity (Figure 1B), thus indicating more selectivity towards MDA-MB-231 drug-resistant human BC cells than MCF-12A normal epithelial breast cells.

Induction of apoptosis by Ambrosin in MDA-MB-231 human drug-resistant BC cells

Apoptosis analysis of Ambrosin-treated MDA-MB-231 human drug-resistant BC cells was performed through AO/EB staining, annexin V/PI

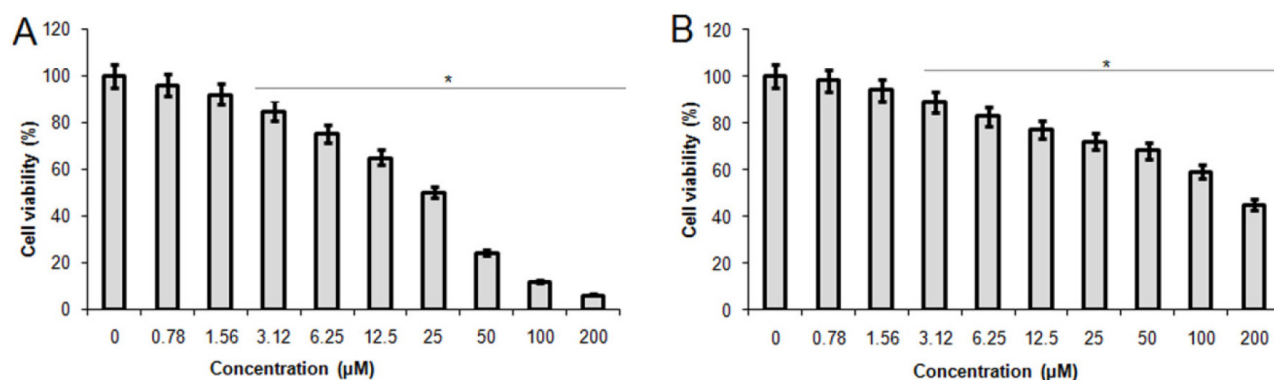


Figure 1. A: Dose-dependent cytotoxic effects of ambrosin on MDA-MB-231 human drug-resistant BC cells as indicated. **B:** Cytotoxic effects of ambrosin on normal MCF-12A epithelial breast cells as indicated. Data are shown as mean \pm SEM of experiments performed in triplicate. * $p < 0.05$.

staining and phase contrast microscopy. The results of AO/EB staining revealed that in controls, uniform green cells were observed while treated cells revealed both early and late stage apoptotic cells marked with yellow-green in case of early stage and asymmetrically localized and concentrated orange nuclei under fluorescence microscope (Figure 2). Furthermore, the number of apoptotic cells increased significantly on increasing the dose of Ambrosin (control, 12.5, 25 and 50 μM) as indicated by annexin V-FITC/PI staining (performed to quantify the apoptotic cell percentage) (Figure 3). In untreated cells the apoptosis induction was insignificant.

Phase contrast microscopy revealed significant morphological changes in cellular structure of MDA-MB-231 human drug-resistant BC cells. Ambrosin treatment led to overall increase in the number of apoptotic cells, cytoplasm shrinkage, formation of apoptotic bodies, reduced cell volume, and chromatin condensation. Control cells retained their identity and dispersed all over the culture plate. Lower drug dose resulted in loss of normal shape and chromatin condensation, while higher doses revealed higher morphological changes indicative of late-stage apoptosis, like round shape, condensation of cytoplasm, condensation of chromatin, cell shrinkage and formation of apoptotic bodies (Figure 4).

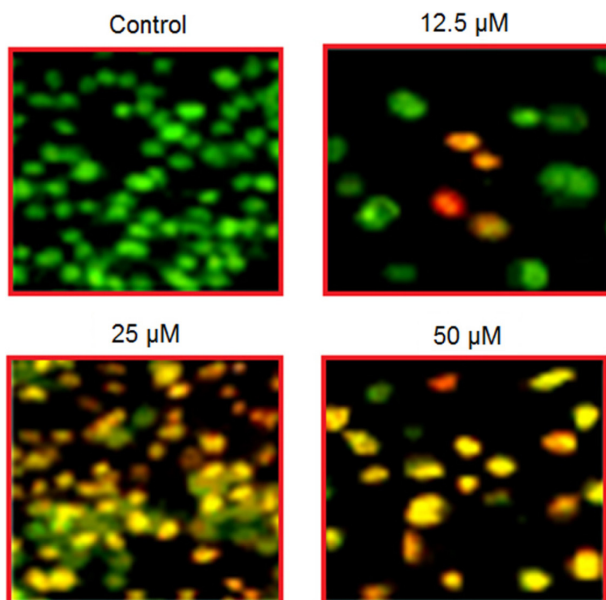


Figure 2. AO/EB staining observations of MDA-MB-231 human drug-resistant BC cells under fluorescence microscope, revealing green fluorescence at zero doses while early and late apoptotic cells were identified with yellow-green and orange nuclei, respectively. The Figure shows that ambrosin induces apoptosis in MDA-MB-231 cells in a dose-dependent manner. Data is collected from three different experiments.

Furthermore, western blotting analysis was performed to evaluate the effect of Ambrosin on apoptosis-associated proteins. The results revealed that it tremendously enhanced the expression of Bax and blocked the expression of Bcl-2 (Figure 5).

Ambrosin inhibits colony formation of MDA-MB-231 human drug-resistant BC cells

Ambrosin's impact on colony formation in MDA-MB-231 human drug-resistant BC cells was

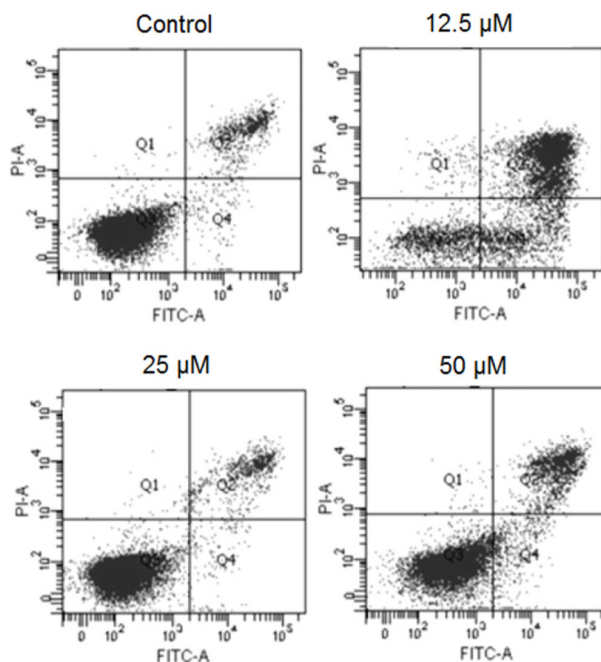


Figure 3. Annexin V/PI staining for apoptotic cell quantification using flow cytometry with increasing dose concentration as indicated in the flow chart. The Figure shows that the percentage of apoptotic MDA-MB-231 cells increased in a dose-dependent manner. The experiments were performed in triplicate.

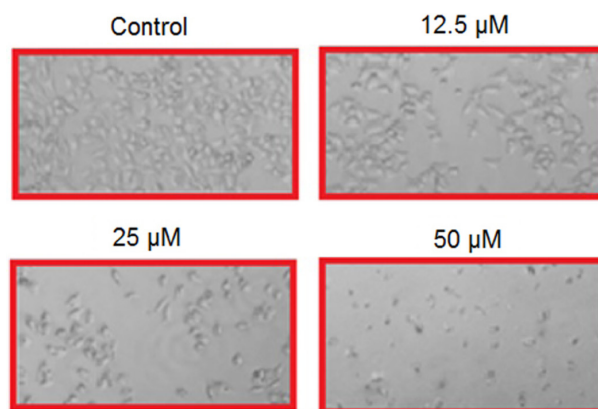


Figure 4. Phase contrast microscopy for morphology determination of treated and untreated MDA-MB-231 human drug-resistant BC cells, indicating cytoplasm shrinkage, formation of apoptotic bodies, reduced cell volume, and chromatin condensation. Data are shown as mean \pm SEM of experiments performed in triplicate.

assessed through clonogenic assay. After treatment with varying drug doses (control, 12.5, 25 and 50 μM), cells were observed under a light microscope (Figure 6). The results revealed that Ambrosin treatment induced inhibition of colony formation in a concentration-dependent manner.

Role of Ambrosin in downregulating MMP and upregulating ROS of MDA-MB-231 human drug-resistant BC cells

The role of Ambrosin in regulating MMP and ROS in MDA-MB-231 human drug-resistant BC cells was determined through flow cytometry. After treatment with varying Ambrosin doses (control, 12.5, 25 and 50 μM), it was observed that MMP started to decrease significantly on increasing the molecule's concentration. MMP at 50 μM of Am-

brosin was reduced to 20%, indicating the current test drug as potential MMP suppressor (Figure 7). Subsequently, ROS of MDA-MB-231 human drug-resistant BC cells was detected and the results suggested a significant increase in ROS percentage after Ambrosin treatment. ROS was 100, 140, 180 and 225% in controls, at 12.5, 25 and 50 μM , respectively (Figure 8). Thus Ambrosin resulted in downregulating of MMP and upregulating ROS levels in a dose-dependent manner.

Ambrosin actively blocked the Akt/ β -catenin signaling pathway in MDA-MB-231 human drug-resistant BC cells

Western blotting analysis was performed to unveil the role of Ambrosin on Akt/ β -catenin signaling pathway in MDA-MB-231 BC cells. After subjecting the cells to treatment with different concentrations (control, 12.5, 25 and 50 μM) of the current test molecule, cells were visualized under Odyssey infrared imaging system. The results revealed that the expression of Akt/ β -Catenin signaling pathway related proteins was altered significantly, with increasing the levels of AKT and GSK-3 β , and decreasing p-AKT and p-GSK-3 β levels in a dose-dependent manner. Hence, it can be concluded that Ambrosin targets Akt/ β -Catenin signaling pathway in a concentration-dependent manner (Figure 9).

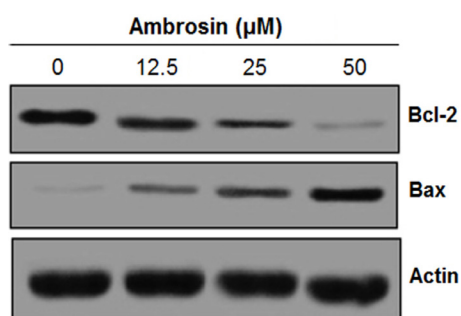


Figure 5. Impact of ambrosin on the expressions of apoptosis-associated protein levels Bcl-2 and Bax, indicating dose-dependent inhibition of Bcl-2 and upregulation of Bax, as indicated. Actin was used as normalisation control. The experiments were performed in triplicate.

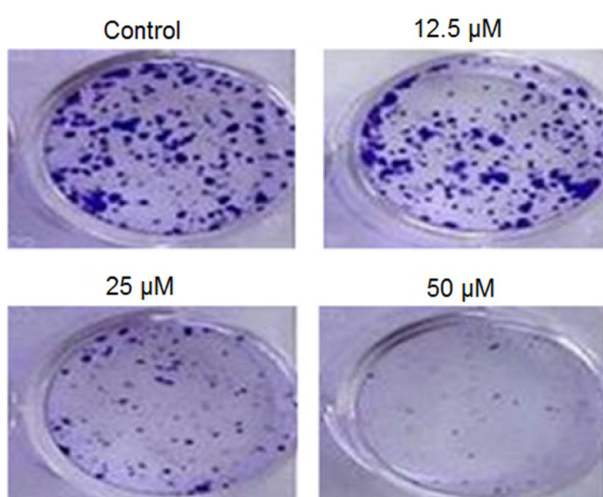


Figure 6. Clonogenic assay for determination of the inhibitory effects of ambrosin on colony formation of treated MDA-MB-231 human drug-resistant BC cells using inverted microscope. The results of the Figure indicate a dose-dependent decrease of colony formation of MDA-MB-231 cells upon ambrosin treatment. The experiments were performed in triplicate.

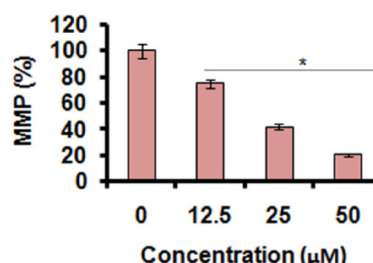


Figure 7. MMP calculations through flow cytometry, after treatment with varying ambrosin concentrations as indicated. Data are shown as mean \pm SEM of experiments performed in triplicate. * $p < 0.05$.

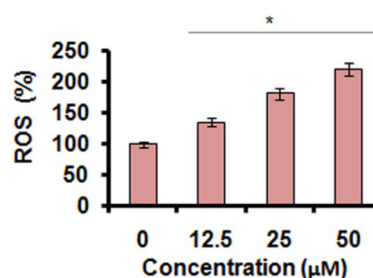


Figure 8. ROS calculations through flow cytometry, after treatment with varying ambrosin concentrations as indicated. Data are shown as mean \pm SEM of experiments performed in triplicate. * $p < 0.05$.

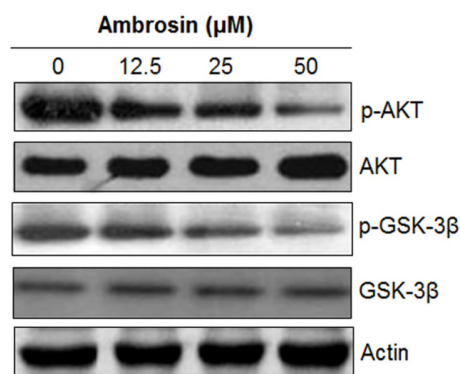


Figure 9. Western blotting analysis to determine the effect of ambrosin on Akt/ β -Catenin signalling pathway through examining the expressions of AKT, p-AKT, GSK-3 β and p-GSK-3 β , as indicated. Actin was taken as normalisation control. The Figure shows that ambrosin inhibits the expression of p-AKT and GSK-3 β . The experiments were performed in triplicate.

Discussion

Breast cancer genesis is due to genetic alterations in normal cells, transforming them into malignant cells [21]. This results in anomalies of cellular processes including cell cycle, angiogenesis and apoptosis [22]. Hence, these pathways serve as potential therapeutic targets in cancer treatment, especially apoptosis. If a cell is found to be damaged, it is discarded/disintegrated through apoptosis, a natural phenomenon of cell death. Apoptosis is mediated via two major pathways: mitochondrial and death receptor pathways, both of them functioning through caspase cascade which eventually results in apoptosis induction [23]. Suppression of apoptosis during carcinogenesis involves three distinctive mechanisms i.e. loss of caspase activity, muddled signalling of death receptors and imbalance among apoptosis-dependent proteins [24]. Thus targeting Bcl-2, caspase cascade and other factors related to apoptosis signalling have become a major strategy in cancer management. In the current study, Ambrosin sesquiterpene lactone was examined for its selective and potent anticancer activity in MDA-MB-231 human drug-resistant BC cells through mitochondrial mediated apoptosis, caspase activation, ROS generation and targeting Akt/ β -Catenin signalling pathway. Cell viability was determined through WST-1 assay revealing significant dose-dependent suppression in cell viability of MDA-MB-231 BC cells as compared to the normal MCF-12A epithelial breast cells. Apoptosis analysis was performed

through AO/EB staining, Annexin V/PI staining, phase contrast microscopy and western blotting analysis. The results showed that the cytotoxic effects of Ambrosin, studied through AO/EB staining, revealed early and late stage apoptotic cells marked with yellow-green in case of early stage and asymmetrically localized and concentrated orange nuclei in late stage, annexin V/PI staining revealed increased number of apoptotic cells on increasing the dose and phase contrast microscopy showed significant morphological changes after varying Ambrosin treatment doses, including cytoplasm shrinkage, formation of apoptotic bodies, reduced cell volume, and chromatin condensation. Western blotting analysis depicted significant suppression in the expression of Bcl-2 and enhanced Bax expressions in a dose-dependent manner. Furthermore, clonogenic assay was performed to determine the impact of the current test molecule on colony formation, with the results revealing significant inhibitory and dose-dependent effect on colony formation. Next, MMP and ROS percentage calculations were performed through flow cytometry, and the results indicated significant decrease in MMP and increase in ROS percentage. Finally, the impact of Ambrosin on Akt/ β -Catenin signalling pathway was assessed through the expressions of associated proteins with western blotting assay, which revealed significant increased GSK-3 β and AKT levels, and decreased p-GSK-3 β and p-AKT levels thus blocking Akt/ β -Catenin signalling pathway.

Conclusions

Using viability, apoptosis, MMP, ROS and Akt/ β -Catenin signalling pathway assessment, it may be concluded that Ambrosin is a potent anticancer agent against MDA-MB-231 human drug-resistant BC cells and thus it may be considered as a potential therapeutic agent.

Acknowledgement

This study was supported by the Scientific Research Project of Human Education Department in 2019 (No.19C1623).

Conflict of interests

The authors declare no conflict of interests.

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